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"Characterization of *Rhodotorula* sp and it's Metabolites for Nutraceutical Applications"

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DEPARTMENT OF STUDIES AND RESEARCH IN MICROBIOLOGY

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CHAPTER I
INTRODUCTION

Industry has become increasingly interested in using and creating non-contaminating or quickly biodegradable chemicals (Falleh, *et al*., 2020; Panwar, *et al*., 2012). One potential substitute is in creating novel bioprocesses that use microbial synthesis of useful metabolites (Pham, *et al*., 2019; Cravens, *et al*., 2019). These metabolites are frequently very large molecules, such as biopolymers and active medicinal ingredients, which are difficult to produce through chemical synthesis. It is possible to use almost any bacteria for microbial synthesis, but not all of them can make valuable and complicated compounds as part of their natural metabolism. Yeasts have an enormous amount of potential among various microorganisms used in bioprocesses because of a complicated and varied metabolism that makes them optimal for synthesizing a wide range of molecules (Alcaino, *et al*., 2015; Papanikolaou, *et al*., 2011). Additionally, yeasts have several benefits, like rapid growth (3-7 days), great resistance to unfavorable culture conditions (such as temperature, pH, and oxygen concentration), and ease of genetic manipulation (Mikkelsen, *et al*., 2012).

The phylum Basidiomycota, Class Urediniomycetes, and Order Sporidiales all contain the genus *Rhodotorula*. It can produce a variety of useful metabolites, many of which are crucial for food industries due to its biotechnological potential and safety implications. It is also a good source of proteins, lipids, enzymes, and vitamins. *Rhodotorula* are unicellular yeasts and are described as being soft, smooth, and moist, occasionally the presence of mucoid nature promotes rapid growth (Hernandez-Almanza, *et al*., 2014).

The most crucial items gained from microbial sources for human requirements are enzymes. Phytase produces myo-inositol and phosphoric acid from the hydrolysis of phytic acid (E.C.3.1.3.8). On a commercial basis, microbial sources of phytase are more promising. For biotechnological applications, microbial phytases are crucial for reducing phytate in foods and feedstuffs (Pandey, *et al*., 2001; Sesirekha, *et al*., 2012). Exopolysaccharides (EPSs) are long, branched chains containing a variety of sugars, primarily rhamnose, glucose and galactose in various quantities. EPS are divided into homopolysaccharides and heteropolysaccharides depending on the repeating sugar units in their monomer composition. EPS such as mannan, glucomannan, glucan, galactomannan and phosphomannan are produced by yeast from the genera *Candida*, *Cryptococcus*, *Rhodotorula*, *Hansenula*, and

Sporobolomyces.

Lipid-soluble pigments called carotenoids are abundant in nature, give many foods attractive colours, and have significant biological functions. They are primarily classified as xanthophyll and carotenes depending on the presence or absence of oxygen atoms in their structures (Mussagy, *et al*., 2021). They are referred to as oleaginous yeasts and can acquire lipids up to 20% of their biomass when cultured under controlled conditions. Triglycerols, which resemble traditional plant oils, make up the majority of the lipids generated by these organisms. Polysaccharides comprise over 75% of the dry cell weight of the yeast cell wall (Mussagy, *et al*., 2021; Miranda, *et al*., 2020). *Rhodotorula minuta* is a well-known producer of metabolites with importance in food production, agriculture, and other industries.

CHAPTER II
REVIEW OF LITERATURE

Chapter II of this thesis comprises an extensive literature survey and includes recent and relevant references cited in Introduction section, methods section wherein the protocols adopted with or without modifications. Discussion on the generated data is also compared with recent and peer reviewed research findings.

Based on the broad survey of literatures and based on the increasing market demand for natural products with various applications, the following objectives were proposed for this doctoral program

OBJECTIVES

1. Isolation and characterization of *Rhodotorula* sps.
2. Strain improvement and optimization of physico-chemical conditions for culturing wild and mutant strains of *Rhodotorula* sps.
3. Characterization of secondary metabolites produced by wild and mutant strains of *Rhodotorula* sps.
4. Food and Therapeutic applications of secondary metabolites produced by wild and mutant strains of *Rhodotorula* sps.

MATERIALS

Glasswares:

All experiments were conducted using glassware of the "Borosil" grade. After soaking in a chromic acid solution (100g/l potassium dichromate diluted in 500 ml of strong sulfuric acid) for two hours, they were cleaned with water. The glasswares were thoroughly cleaned with distilled water, wiped and sterilized before use.

Chemicals used:

Every chemical utilized as an analytical reagent (AR) & HPLC grade is obtained from Himedia, Merck and Sigma, India and other leading scientific companies. Such as Potato Dextrose agar, Yeast Malt Extract agar, Malt Extract Media, Glucose Yeast Peptone Mannitol agar, Sabouraud Dextrose agar, Fat-producing medium, beef extract, bacteriological peptone, yeast extract, malt extract, monobasic sodium phosphate, starch, potassium nitrate, potassium dihydrogen phosphate, magnesium sulphate, glucose, maltose, fructose, lactose, xylose, sucrose, urea, ammonium sulphate, sodium nitrate, ammonium nitrate. Acrylamide, bisacrylamide, tris HCl, Sodium Dodecyl sulphate (SDS), Ethylene diamine tetra acetic acid (EDTA), Tween- 80, ammonium persulphate (APS), Coomassie Brilliant blue G 250, N, N, N', N'- tetramethyl ethylene diamine (TEMED), bromothymol blue, N, N, N', N'- tetramethyl-p-phenylenediamine (TMPD), hydrogen peroxide, bile salts, esculin, ferric citrate, potassium iodide, sodium hypochlorite, ammonium sulphate, sodium hydroxide, potassium ferric cyanide, trichloroacetic acid, ferric chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, sodium phytate, ammonium molybdate, sulphuric acid, acetone, phytase standard, hydrochloric acid, ethylmethane sulphonate (EMS), sodium thiocyanite, potassium dichromate, chloroform, acetic acid, potassium iodide.

Sterilization of glass wares:

For two hours, at 180°C in a hot air oven, all the glasswares were sterilized. An autoclave was used to sterilize all the growth media and distilled water blanks for 20 minutes at 15 lbs pressure. A laminar airflow chamber was used for microbiological operations such as isolation, purification, and inoculation.

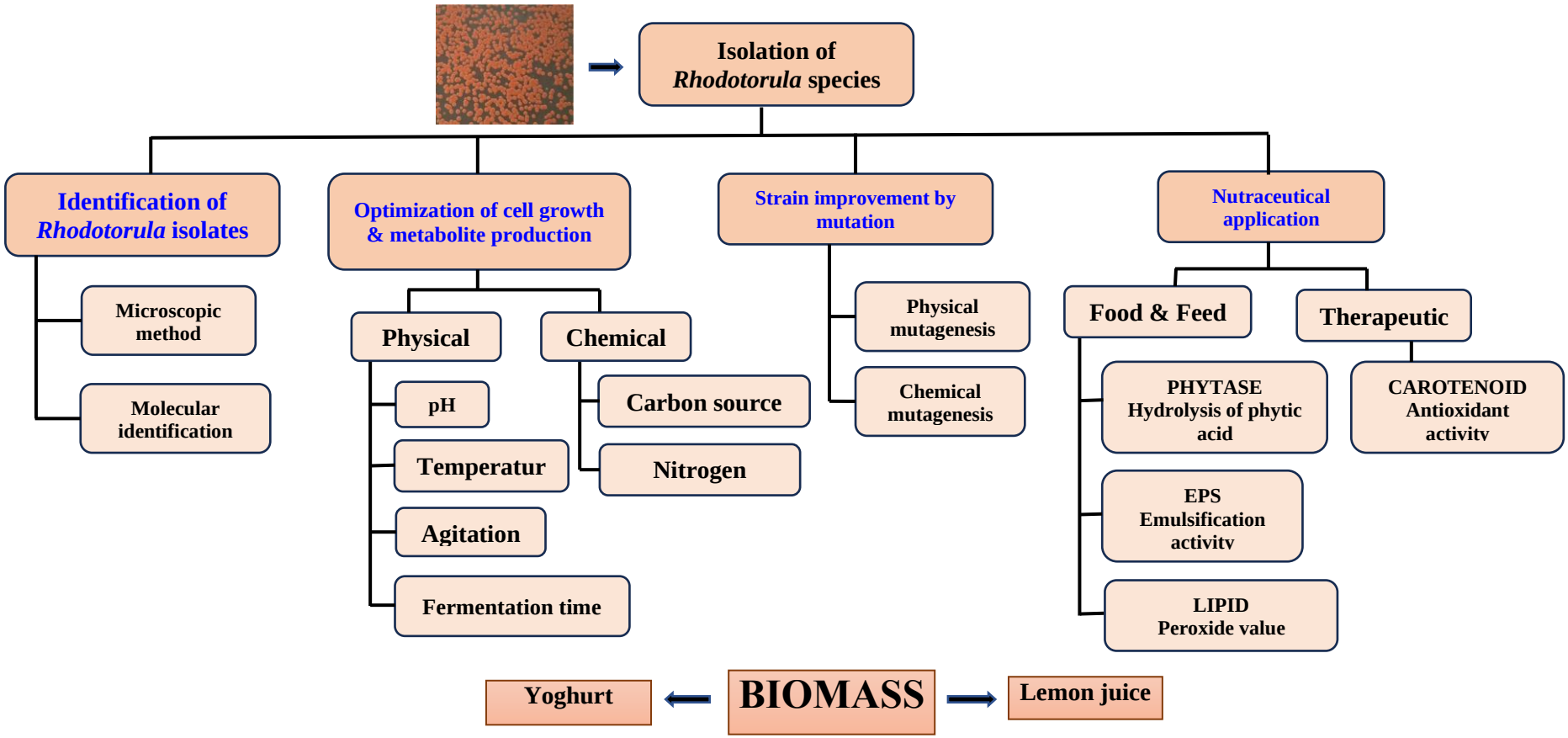

Fig 1: Flow chart of the study

METHODOLOGY

OBJECTIVE I: ISOLATION AND CHARACTERIZATION OF *RHODOTORULA* SPECIES

One hundred and eighteen heterogeneous samples agricultural soils (35), milk (35), compost soil (10), air (5), flowers (9), leaves (9), dairy products (2), and fruits (13) were collected in and around Kodagu district of Karnataka state, INDIA. The samples were serially diluted and appropriate dilutions were plated on MYE agar medium and incubated for three days at 30°C (Haelewaters et al., 2021). Discrete colonies resembling yeasts were isolated and stored on MYE slants at 4˚C for further work. Fourteen pigmented yeast isolates were obtained from natural sources based on their physical and biochemical traits. The biochemical tests included ethanol production, catalase, bile esculin, oxidase, sugar and nitrate assimilation tests (Nafady et al., 2019; Begum et al., 2020).

Molecular identification was carried out by investigating morphological and physiological characteristics of the yeast isolates using methodologies of Zhao et al., (2019). rDNA partial sequence analysis was performed using ITS1 and ITS4 PCR primers. The isolate's genetic code was obtained using CTAB kit. DNA purity and amount were evaluated. PCR was performed at -20°C, and ITS segments were aligned using Clustal W. A phylogenetic tree was constructed using MEGA 11 and the neighbour-joining technique.

The isolated yeast strains were screened for pigment production by growing the strains in malt yeast extract agar (MYEA) slants for 5 days, and carotenoid content in the supernatant was measure using β-carotenoid as a standard. The highest pigment-producing isolate was chosen for further analysis (Arulselvi et al., 2013).

The isolates were also screened for phytase production by culturing them for five days in MYE broth and the supernatant was used to measure crude extracellular phytase activity using sodium phytate as a substrate (Heinonen and Lahti, 1981). One unit of phytase is the amount of enzyme required to release 1 μmol of inorganic phosphate from the substrate under standard conditions. The specific activity of phytase enzyme was determined by estimating total protein concentration in the samples using the Bradford protein assay method, using UV spectrophotometer (He, 2011; Nkoi et al., 2022).

OBJECTIVE 2: STRAIN IMPROVEMENT AND OPTIMIZATION OF PHYSICO-CHEMICAL CONDITIONS FOR CULTURING WILD AND MUTANT STRAINS OF *RHODOTORULA* SPS.

The study investigated the impact of temperature, pH, agitation, and fermentation time on phytase, carotenoids (intra and extracellular), by cultivating the isolate in MYE broth incubated at various temperatures, pH levels, and fermentation duration. The conditions were kept constant at 5.5, 35°C, and 5 days. The broth was centrifuged at 6000 rpm and 4°C for 20 minutes, and the supernatant was collected for phytase (Heinonen & Lahti 1981), extracellular carotenoids assay. Cell pellets were dried overnight, heated with 0.1N HCl, and then chilled. The mixture was centrifuged for 10 minutes to extract the intracellular carotenoids, and absorbance was measured at 450 nm using acetone as a solvent (Yadav and Prabha, 2014).

Cell pellets were collected and dried for lipid extraction using the Soxhlet method, and samples were rotary evaporated at 50°C (Kot, et al., 2017). The culture supernatant was collected and the extracellular polymeric substance (EPS) was precipitated using cold isopropanol. The precipitate was collected, washed twice, dried, and weighed (Kanamarlapudi and Muddada, 2017).

The isolate was cultured in MYE broth to assess the impact of different carbon and nitrogen sources on phytase, intracellular and extracellular carotenoids, lipids and exopolysaccharides. The experiments were carried out by the methods given in earlier section of this synopsis.

Rhodotorula minuta was subjected for physical and chemical mutagenesis to improve the strain. The native strain grown in MYE medium was centrifuged at 6000 rpm for 15 minutes at 4°C and the resulting pellets were suspended in sterile distilled water. One ml aliquot of the cell suspension was then taken in sterile Petri plates and were exposed to UV radiation for varying time intervals of 5, 10, 15, and 20 minutes within a UV chamber set at 280nm. For chemical mutagenesis, the culture pellets obtained were treated with different concentrations (3, 6 and 9 mM) of Ethyl Methane Sulphonate solution for 1 hour. Following treatment, the suspension was centrifuged at 6000 rpm and washed with sterile distilled water. All tubes were then kept at 4°C overnight for DNA repair. Finally, 0.1 ml of inoculum from each tube of both physical and chemical mutagenesis was spread using the spread plate method and incubated at 30°C for 3 days (Shivanna and Govindarajulu, 2009). Sixteen isolates from UV irradiation and three from EMS-treated wild strain of *Rhodotorula minuta* were obtained based on their

morphological variations including colony size and colour and were maintained in MYE agar media.

All the mutant strains were screened for metabolite production such as Phytase, Carotenoids, Lipids and Exopolysaccharides by following the methods mentioned earlier. Optimization of physical parameters on mutant strains of *Rhodotorula minuta* was studied for metabolites such as phytase, carotenoids, lipids and exopolysaccharides by following the methods mentioned earlier.

Optimization of culture media on the growth of mutant *Rhodotorula minuta* strains was carried out by inoculating CMR1, CMR2, CMR3, MR5, MR7, MR9 and MR13 in different agar media such as PDA (Potato Dextrose Agar), ME (Malt Extract), MYE (Malt Yeast Extract), GYMP (Glucose Yeast Malt Peptone), FPM (Fat Producing Medium) and SDA (Saboroud's Dextrose Agar). All the inoculated plates were incubated for 3 days at 30°C.

OBJECTIVE 3: CHARACTERIZATION OF SECONDARY METABOLITES PRODUCED BY WILD AND MUTANT STRAINS OF *RHODOTORULA* SPS.

The separation of a crude phytase enzyme from submerged fermentation marked the beginning of partial purification of the enzyme. Every step was carried out at 4°C. The crude enzyme was fractionated using ammonium sulphate salt as per the standard chart. Solid ammonium sulphate was gradually added to the crude enzyme solution by continuous stirring until it reached 90% saturation. The precipitate was collected after centrifugation for 20 minutes at 10,000 rpm. The precipitate was dissolved in a small volume of 0.2 M acetate buffer (pH 5.5) followed by dialysis using 0.2 M acetate buffer. After dialysis, the samples underwent total protein estimation before being loa ded onto a 10% SDS-PAGE gel under denaturing conditions, following the method outlined by Laemmli (1970). Subsequently, the protein bands within the gel were visualized post-staining with Coomassie Brilliant Blue R-250.

The carotenoids extracted from both wild and mutant strains of *Rhodotorula minuta*, were characterized by liquid chromatography-mass spectrometry, using a C18 reversed-phase column and a 4000 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer. The resulting compounds were identified using Mnova-10 software for molecular mass, formula, and IUPAC name.

Rhodotorula minuta strains were grown in an optimized medium to produce lipids.

Lipid estimation was performed using trans-esterification to create fatty acid methyl esters (FAMEs). Total fatty acid compositions were extracted using petroleum ether, dissolved in hexane, and incubated with methanolic KOH. The upper phase was collected and analyzed using GC-MS (Kot, et al., 2017). The FAME fraction of the hexane layer was separated using a MassHunter gas chromatography-mass spectrometry, using an SPB 1 capillary column and nitrogen as a carrier gas, with operating temperatures set at 230 and 260°C.

The optimized media was inoculated with wild and mutant *Rhodotorula minuta* strains, and the fermented broth was examined for exopolysaccharide yield. The microstructure and surface morphology were examined using a scanning electron microscope at 20 keV (Kanamarlapudi and Muddada, 2017). The elemental configuration of EPS was determined via Energy Dispersive X-ray analysis. The Fourier Transform was applied to EPS, and the infrared spectrum of the polysaccharide sample was analyzed using a PerkinElmer Spectrum spectrometer. Compressed discs were created, and the spectrum adjusted for KBr background. The pellets were then scanned using 32 scans in the 4000–500 cm^{-1} range at a resolution of 4 cm^{-1} (Kanamarlapudi and Muddada, 2017).

OBJECTIVE 4: FOOD AND THERAPEUTIC APPLICATIONS OF SECONDARY METABOLITES PRODUCED BY WILD AND MUTANT STRAINS OF *RHODOTORULA* SP.

Therapeutic applications of carotenoids and exopolysaccharides were studied through DPPH Free Radical Scavenging Activity Assay by using a modified method of Blois's 1958. The extracts were combined with a methanolic solution of DPPH at different concentrations, and absorbance was measured. The half-maximal inhibitory concentration (IC_{50}) was determined using the linear regression equation $y = mx + c$, and the values were obtained using the viability graph (Adeniran et al., 2020). The proportion of scavenging activity of each extract on the DPPH radical was calculated as % inhibition of DPPH.

Antibacterial activity of Exopolysaccharide from both wild and mutant strains of *Rhodotorula minuta* was studied against various pathogenic microorganisms by agar well diffusion assay. The test bacterial strains were spread plated on Nutrient Agar medium and left for few minutes. EPS powder was dissolved in sterile distilled water at different concentrations and aseptically added into the wells. The plates were incubated for 24 hours at 37°C, and the diameter of the inhibitory zone was determined using the Hi-media zone scale (Gowthami et

al., 2021).

The lipids obtained from both wild and mutant strains of *Rhodotorula minuta* were subjected for food application by the Determination of Peroxide value. The reactive oxygen levels reported in milliequivalents (meq) of free iodine per kilogram of fat are known as the peroxide value (POV). Titrating potassium iodide-released iodine with sodium thiosulphate solution yields the result (Longobardi et al., 2021).

Exopolysaccharide extracted from *Rhodotorula minuta* wild and mutant strains were also tested their emulsification activity. Several vegetable oils including rice bran oil, almond oil, coconut oil, sunflower oil and olive oil were used for the study. After adding around 1.5 mL of vegetable oil to 1.5 mL of EPS at different concentrations (0.5, 1.0, and 1.5 mg/mL), the mixture was strongly vortexed for two minutes and the emulsification activity (% EA) was assessed after just one hour, while the emulsion stability was measured as the emulsification index (% EI) after 24, 48, and 72 hours (Kanamarlapudi and Muddada, 2017).

The wild culture of *Rhodotorula minuta* was applied for food formulation such as yoghurt and lemon juice. The strain was cultivated in a malt yeast extract medium, the culture broth was centrifuged for 15 minutes at 6000 rpm and 4^{o}C after incubation. The pellets were collected and washed with sterile distilled water twice and the cells were dried in an hot air oven at 60^{o}C overnight. After drying, the cells were collected and weighed. Fifty ml of lukewarm milk was dispensed in a beaker and dry cell biomass of *Rhodotorula minuta* with different concentrations (0.5% and 1.0%) was added to the milk. The milk was left to be incubated overnight at room temperature. After incubation, the samples were observed for coagulation and compared with control sample prepared without the biomass. The formulated yogurt was subjected for estimation of lactic acid by titrating against 0.1 N NaOH using phenolphthalein indicator.

Lemon juice formulation was prepared by dissolving 36 g of table sugar in 100ml of potable water, and 1 tablespoon of lemon syrup was added in 2 sets. To the lemon juice, 0.5 and 1.0% of dry cells of *Rhodotorula minuta* wild strain were added. The samples were left for 10-15 minutes at room temperature, observed for colour release in the juice and compared with a control sample that did not contain any biomass.

CHAPTER IV
RESULTS AND DISCUSSION

OBJECTIVE 1
ISOLATION AND CHARACTERIZATION OF *RHODOTORULA* SPECIES

Fourteen yeast isolates were identified based on colony morphology, cell shape, and budding pattern from 108 heterogeneous samples. All isolates grew on tested sugars except G10, G13, and G14, showed positive results in nitrate assimilation, and produced ethanol.

The study focused on purifying isolates of *Rhodotorula* yeast strains, known for producing valuable carotenoid pigments. Only 14 strains were isolated and cultivated in Malt Yeast Extract broth. The supernatant was assayed for extracellular pigment production, with orange-colored pigments absorbed maximally at 450-500nm. Initial phytase screening revealed variations between isolates, with the maximum activity found in isolate G11 at 400nm.

The isolated yeast cells were identified as *Rhodotorula mucilaginosa* (05), *Rhodotorula evergladensis* (01), *Rhodotorula araucariae* (01), *Rhodotorula glutinis* (01), *Rhodotorula minuta* (01), *Rhodotorula graminis* (01) and *Rhodotorula toruloides* (01) by molecular sequencing method and three others were identified as *Candida rugosa* that have been reported as opportunistic pathogens.

OBJECTIVE 2:
STRAIN IMPROVEMENT AND OPTIMIZATION OF PHYSICO-CHEMICAL CONDITIONS FOR CULTURING WILD AND MUTANT STRAINS OF *RHODOTORULA* SPS.

The study found that phytase production and cell biomass from *Rhodotorula minuta* through physical parameters like temperature, pH, RPM and fermentation time, revealing a maximum activity of 91.85, 27.13, 18.9 and 21.4 U/ml at 35°C, pH of 5.5, 150 rpm and 120 h respectively. The optimum temperature for phytase activity in *Cyberlindnera jadinii* CJ2 was found to be 50°C with the highest activity of 51.95 mU/mgdw, while the activity decreased with an increase in temperature (Capusoni et al., 2021). Research indicates that pH 4.5 is optimal for phytase activity in *Cyberlindnera jadinii* CJ2, *Muscodor* sp., and *Kodamea ohmeri* BG3 (Capusoni et al., 2021; Alves et al., 2016). The study reveals that 125 rpm is the optimal agitation speed for phytase production from *Muscodor* sp., while 170 rpm yields the highest phytase from marine yeast *Kodamea ohmeri* BG3 (Alves et al., 2016; Li et al., 2008). Studies show that *Aspergillus niger* is influenced by the incubation period, with the fourth day showing

highest growth and phytase levels (Sandhya et al., 2015).

The wild *Rhodotorula minuta* strain was used for carotenoid production at varying physical parameters such as temperatures, pH, RPM and fermentation time and found to be 0. 495 µg/ml and 2.75 µg/g; 0.57 µg/ml and 3.185 µg/g; 0.328 µg/ml and 2.011 µg/g; 0.301 µg/ml and 2.673 µg/g at 25°C, pH of 5.0, 200 rpm and 108 h respectively. Dyaa et al. (2022) optimized physical and chemical conditions for carotenoid generation and cell growth in *Rhodotorula* sp. strain ATL72 at 27.5°C and found to be 25.14 mg/L. Naz et al. (2023) found that pH affects carotenoid production during fermentation, affecting cell development and pigment biosynthesis. The optimal pH varies with the fungal species and growing media, with limited cell development and unfavorable carotenoid synthesis. Machado et al., (2019) found that 130 RPM is the optimal agitation speed for both biomass and carotenoid production in *Rhodotorula mucilaginosa* URM 7409. The study found that pigment output reached its peak after 120 hours of shaker culture incubation, with growth entering the stationary phase and pigment production continuing for 120 hours (Allahkarami et al., 2021).

In the present study lipid contents were found to be 368, 208, 59 and 51 mg/g with temperature, pH, RPM and fermentation time of 30°C, 5.0 pH, 150 rpm and 36h respectively. The exopolysaccharide contents were found to be 2.3, 20, 3.2 and 0.8 mg/ml at 20°C, 6.0 pH, 150 rpm and 102h of temperature, pH, RPM and fermentation time respectively. According to Osman et al., (2022), the optimal temperature for lipid production from *Rhodotorula* sp. is 30°C, with a maximum experimental response of 5.09 g/L at this temperature. Understanding the interplay between pH and other environmental factors is crucial for optimizing lipid production in *Rhodotorula* cultures for various industrial applications (Jiru et al., 2017; Osman et al., 2022). Another study found that *Morchella esculenta* SO-02 grew at pH 6.5, with the highest DCW and EPS concentration. *Rhodosorus* sp. SCSIO-45730 had the highest polysaccharide yield, while *Rhodotorula glutinis* produced the highest EPS at a pH of 6.0 (Wang et al., 2022; Ghada et al., 2012). Research shows *Rhodotorula diobovata* and *Rhodotorula mucilaginosa* produce lipids at varying agitation speeds, with the latter showing the highest lipid accumulation at 200 rpm, with maximum biomass and lipid yields of 17.71 g/L and 8.15 g/L respectively (Osman et al., 2022; Gedela et al., 2023). (Mohammed et al., 2021) have shown that incubation time significantly impacted YMM19 growth and EPS production, with maximum growth occurring after 5 days and EPS production decreasing after 7 and 9 days.

The study optimized the phytase production from *Rhodotorula minuta* using various carbon and nitrogen sources, with sucrose and ammonium sulphate showing the highest phytase activity (26.33 and 50.12 U/ml). Glucose has the highest biomass of 0.348 g/100ml while ammonium sulphate showing the maximum biomass of 0.288 g/100mml. Li et al., (2008); Kanti et al., (2020) found that glucose (30.0 g/l) is the best carbon source for phytase production in marine yeast *Kodamea ohmeri* BG3, with isolates *Candida natalensis, Saturnispora quitensis*, and *Candida albicans* showing the best activity. Nitrogen source affects phytase production from *Candida albicans* with yeast extract, while *Candida natalensis* and *Saturnispora quitensis* show the highest activity with ammonium sulfate (Kanti et al., 2020).

The study examines total carotenoids from the wild *Rhodotorula minuta* strain using various carbon and nitrogen sources. The results showed the highest carotenoid content of 0.339 µg/ml (EC) and 2.194 µg/g (IC) of dry cells with Fructose. While ammonium sulphate and urea produced the highest total carotenoid found with ammonium sulphate (0.303 µg/ml & 2.341 µg/g) and urea (0.259 µg/ml & 3.205 µg/g). Research on microbial fermentations reveals glucose and yeast extract as the best carbon and nitrogen source for carotenoid production at 962 µg/l, while fructose have been shown as the best carbon source (Zhao et al., 2019).

The study found that lactose and sodium nitrate were found to be the best carbon and nitrogen sources for maximum biomass of 833 mg/g of dry cell and 86 mg/g of lipid respectively. Kongruang et al., (2020) found that molasses is a valuable industrial carbon source, with *Rhodotorula toruloides* TISTR 5154 having the highest lipid content (32.35%), followed by *Yarrowia lipotica* 5054 and *Yarrowia lipotica* 5151. Research on organic nitrogen sources in molasses medium found maize steep liquor as the most effective for lipid synthesis, with a yield of 8.27g/L (Osman et al., 2022).

The study optimized various carbon and nitrogen sources for exopolysaccharide production from *Rhodotorula minuta*, finding fructose and potassium nitrate as the most effective producing 9 and 6.5 mg/ml respectively. Khani et al., (2016) stated that increasing glucose concentration stimulates EPS synthesis. Microbial EPS is determined by the type of carbon source and yeast species. The study examined the impact of nitrogen sources on the exopolysaccharide production by *Rhodotorula* species. Organic nitrogen has been shown to activate *Rhodotorula minuta* ATCC 10658, resulting in maximum EPS synthesis in soya bean

and yeast extract (Samadlouie et al., 2020).

Strain improvement of *Rhodotorula minuta* by physical and chemical mutagenesis resulted in 16 and 03 putative mutant strains of *Rhodotorula minuta* through UV and EMS treatment respectively. The isolates were successfully sub-cultured on MYE agar plates. Of the 16 mutant strains obtained through UV mutagenesis were screened for phytase, carotenoids, lipids, and exopolysaccharide production. The highest phytase, total carotenoid, lipids, and exopolysaccharide content were found in MR7, MR13, MR5, and MR9. CMR1 strain obtained through EMS treatment showed maximum phytase, carotenoid, and lipid production while highest EPS production was found to be by CMR2.

The study aimed to optimize phytase production through temperature and pH from mutant strains of the *Rhodotorula minuta* strains. Results showed wild strain had the highest activity at 35°C, while putative mutants CMR1 and MR7 showed maximum activity at 30°C and 40°C, respectively. While the maximum activity at a pH 5.5, 7.0 and 6.5 with wild strain, CMR1 and MR7 respectively. Haq et al. (2022) found that chemical mutagenesis improves *Aspergillus oryzae* strains, with maximum phytase activity achieved at 30°C, while more phytase production occurs at 35°C in mutant strains. The data are shown in earlier reports that, the phytase contents of mutant strains of *Pichia kudriavzevii* TY13wt and TY1322 showed the two optimum pHs at 3.5 and 5.5 (Qvirist et al., 2017).

The study examined the impact of temperature and pH on the production of total carotenoids from wild and putative mutants of *Rhodotorula minuta*. Results showed that CMR1 and MR13 had highest carotenoids at 25 and 20°C respectively, while pH 6.5 was optimum for both CMR1 and MR13 strains. *Rhodotorula* sp produced carotenoids of 298 ± 12 µg/g dry biomass in YM broth and 5.3 g/L in *R. mucilaginosa* UANL 001L (Torres-Alvarez et al., 2022). The study found that mutant MM produced maximum carotenoid yields at pH 6.0, while the ideal pH for the terrestrial yeast *Rhodotorula mucilaginosa* to produce total carotenoid was 7.0 (Cong et al., 2007).

The present study analyzed the lipid content of wild and mutant strains of *Rhodotorula minuta* at different temperatures and pH. Results showed that CMR1 and MR5 had higher lipid content at 25°C, while pH 7.5 and 6.5 showed optimum for CMR1 and MR5 respectively. *Rhodotorula mucilaginosa* UANL 001L cells was found to be highest (455±25.17 mg), with a lipid content ranging between 1.5 and 3 g/L (Torres-Alvarez et al., 2022). Another study aimed to enhance lipid accumulation in *Rhodosporidium toruloides* by creating mutant strains with

increased lipid productivity and yield, specifically R-ZL2 and R-ZY13 (Guo et al., 2019).

The present study used MYEB medium to grow wild and putative mutants of *Rhodotorula minuta* for exopolysaccharide (EPS) biosynthesis. CMR2 and MR9 showed the highest production at 40°C, while pH 6.0 and 6.5 was optimum for CMR2 and MR9 respectively. Ghada et al., (2012) and Gientka et al., (2016) found that *Rhodotorula glutinis, Candida, Rhodotorula minuta* and *Cryptococcus flavus* all produce EPS at optimal conditions, with the *Candida* strain producing 0.4-2.4 g/L. Okoro et al., (2021) studied EPS production by *Rhodotorula mucilaginosa* sp. GUMS16 using response surface methodology, finding low pH values favorable but highly acidic conditions leading to excessive production.

In the present study, the mutant strains of *Rhodotorula minuta* were grown on various agar media, finding that the MYE medium was the most effective for growth within 24 hours, compared to other media that took more than 3-4 days. Frengova et al. (2004) and Cutzu et al. (2013) studied mutant strains of *Rhodotorula rubra* GED8 and *Rhodotorula glutinis*, resulting in pigmented cultures at 25°C and 48 hours.

OBJECTIVE 3

CHARACTERIZATION OF SECONDARY METABOLITES PRODUCED BY WILD AND MUTANT STRAINS OF *RHODOTORULA* SPS.

The partially purified phytase enzymes of both wild and mutant strains of *Rhodotorula minuta* correspond to molecular weight 40 kD of standard phytase enzyme. All the samples were partially purified at 30-90% saturation and dialyzed. Yu et al., (2015) and Huang et al., (2009) purified a phytase from *Rhodotorula mucilaginosa* JMUY14, revealing that its activity decreases with temperature rise above 40-60°C.

The crude carotenoid extracts of both wild and mutant strains of *Rhodotorula minuta* were subjected to LC-MS analysis to obtain the details of the components present in pigment. A total of 49 different components and isotopes of the compounds were identified in the pigment of wild strain. The putative mutant strains such as MR13 and CMR1 when subjected to LC-MS analysis revealed a total of 23 and 16 components respectively. Sriputtha et al., (2023) have reported the molecular weight of the purified β-carotene by LC-MS analysis. The chemical composition of four fractions from *Rhodotorula taiwanensis* was analyzed using LC-MS. β-carotene, β-cryptoxanthin, torulene, and torularhodin were identified by their molecular formulae, with β-carotene and β-cryptoxanthin having the molecular formula of $C_{40}H_{56}$ and $C_{40}H_{56}O$ respectively (Parasar et al., 2020).

The details of fatty acid components present in lipid samples of both wild and mutant strains of *Rhodotorula minuta* were obtained by transesterification (FAMEs) followed by GC-MS analysis. 31 esters of fatty acids were obtained from the wild strain while putative mutant strains of MR5 and CMR1 showed 22 and 20 fatty acids esters respectively. Santos et al., (2021) reported that lipids constituted saturated and unsaturated fatty acids from *Rhodotorula toruloides*, with octadecenoic acid, octadecanoic acid and hexadecenoic acid as main constituents. Cajka et al., (2016) have reported the extracellular lipids of hydroxy fatty acids from *Rhodotorula babjevae* UCDFST 04-877 by GC-MS.

Scanning electron micrographs revealed the three-dimensional structure of *Rhodotorula minuta* EPS, characterized by irregular lumps and a coarse surface. The EPS is porous and spherical at higher magnification, with elements including carbon, oxygen, nitrogen, and chlorine. The EPS from *Lactobacillus plantarum* have been observed to have a porous web-like structure (Wang et al., 2015). SEM micrographs of EPS from *Lactobacillus fermentum* CFR2195 have been shown to be flake-like structural units and were highly compact (Yadav et al., 2011).

FTIR is an effective technique that works on the principle that a group of bonds vibrates at characteristic frequencies. It can be employed to detect functional groups and to characterize covalent bonding. The wild strain of *Rhodotorula minuta* showed functional group of hydroxyl and carbonyl group. While putative mutant strains of MR9 and CMR2 showed methyl, carbonyl and amine groups as functional group. Kumar et al., (2011) have shown that broad stretching vibration of hydroxyl groups is characteristic of a carbohydrate ring.

OBJECTIVE 4

FOOD AND THERAPEUTIC APPLICATIONS OF SECONDARY METABOLITES PRODUCED BY WILD AND MUTANT STRAINS OF *RHODOTORULA* SPS.

- The extracted metabolites from wild and mutant strains of *Rhodotorula minuta* were subjected to various tests to check their nutraceutical potential.
- The wild strain exhibited a DPPH radical scavenging activity with IC_{50} value of 124.04 µg/ml, while, MR13 and CMR1 strains showed values of 46.70 and 71.72 µg/ml, respectively which is highly significant.
- EPS obtained from both wild and mutant strains (MR9 and CMR2) did not shown any positive results when tested for antioxidant and antibacterial activity.

- The exopolysaccharides of wild *Rhodotorula minuta*, MR9 and CMR2 were capable of stabilizing emulsions of different vegetable oils (sunflower oil, coconut oil, rice bran oil, olive oil and almond oil).
- The emulsification activity was higher in olive oil with 70% (1%) and 90.90% each with 1 & 1.5% in wild and CMR2 strain while it was found to be 86.95% for coconut oil (0.5%) with EPS of MR9.
- The emulsification index of exopolysaccharide was higher in sunflower oil with (75, 80 & 63.63%) after 24, 48 & 72 h respectively.
- Lipids with a peroxide value (POV) ≤ 10 meq/kg are considered fresh. In the present study, the lipids extracted from both wild and two mutant strains (MR5 and CMR1) showed peroxide values of 2.28, 5.88, and 1.30 meq/kg respectively. After 2 months of storage, they showed 19.6, 26.13, and 6.53 meq/kg respectively. The lipids/ oil obtained from CMR1 continued to be fresh even after 2 months of storage.

An attempt was made to see the applicability of the biomass of *Rhodotorula minuta* in two different food items, such as yoghurt and lemon juice. To enhance the appearance and acceptability of the products, the biomass of *Rhodotorula minuta* was added. The dry cells of *Rhodotorula minuta* were mixed in different concentrations (0.5 and 1.0%) in yoghurt and lemon juice. As per the panelist's feedback both the products were appealing and acceptable without any adverse observations. The extracted carotenoid was identified by HPLC and it was used as a coloring agent for sweet candy jelly preparation (Elsanhoty et al., 2017). The coloring of foods in the next millennium is likely to be characterized by a continued shift towards natural, sustainable, and functional options, driven by consumer preferences, technological advancements, and regulatory considerations.

SUMMARY AND CONCLUSION

Rhodotorula species, a red yeast has generated significant interest due to its potential for nutraceutical applications. The present study focused on isolating *Rhodotorula* sp., from heterogenous samples from the Kodagu region of Karnataka state, INDIA. Selected isolates of *Rhodotorula minuta* could produce several metabolites, including phytase, carotenoids, lipids and exopolysaccharides. UV and EMS mutagenesis carried out the strain improvement for metabolite production followed by optimization of physico-chemical conditions for culture growth and metabolite production from both wild and mutant strains of *Rhodotorula minuta*. The nutraceutical application of all the metabolites showed the best results on antioxidant activity, peroxide value and emulsification activity for carotenoid, lipids, and EPS respectively. The mutant strains such as MR13, CMR1 and MR9 showed better results when compared to other mutants. All the tested metabolites hold promising roles for various health benefits thus making *Rhodotorula minuta*, a valuable candidate for nutraceutical development. Further efforts in research and development are warranted to explore the full range of health-promoting properties associated with *Rhodotorula minuta.* In conclusion, *Rhodotorula minuta* stands out as a promising candidate for the development of novel nutraceuticals aimed at improving human health and well-being.

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RESEARCH PUBLICATIONS:

1. **Gowthami, G. A.,** and Gunashree, B. S. (2023). Isolation, characterization and optimization of *Cystobasidium minutum* for phytase production. Biomedicine. 43(01): 329-34. (Impact factor: 0.1 Elsevier Indian Citation Index)
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BOOK CHAPTERS:

1. **Gowthami, G. A.,** and Gunashree, B. S. (2023). Phytase in Combating Nutritional Implications of Phytates. In the book entitled, "Advances in Enzymology: Role in Industrial and Pharmaceutical Applications", P.K Publishers, New Delhi. ISBN: 978-93-92239-51-9.
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PAPER PRESENTATIONS IN NATIONAL/ INTERNATIONAL CONFERENCE:

1. **Gowthami G. A**., and Gunashree B. S., (2021). Presented **e-poster** entitled **"Characterization of Native and Mutant Strains of *Rhodotorula gracilis* for Phytase production"** in the 3 days International Virtual Conference on Recent Trends in Biomedical Research, held on March 12th to 14th, organized by the Indian Association of Biomedical Scientist, Kerala, India.

2. **Gowthami G. A**., and Gunashree B. S., (2022). A **Poster** was presented on the topic entitled **"Effect of pH and Temperature on the production of phytase from *Rhodotorula* sps."** in the 3 days International Virtual Conference on Emerging and Adoptable Technologies for Sustainable Agro-Food Industries and Economy (EAT-SAFE), held on 20th to 22nd Jan organised by Association of Food Scientists & Technologists (India), CSIR-CFTRI campus, Mysore- 570020, India.
3. Sushmitha M., **Gowthami G. A.,** and Gunashree B. S., (2022). A **Poster** was presented on the topic entitled **"Physico- Chemical Characterization of *Rhodotorula* isolates for Lipid and Exopolysaccharide production"** in the 3 days International Virtual Conference on Emerging and Adoptable Technologies for Sustainable Agro-Food Industries and Economy (EAT-SAFE), held on 20th to 22nd Jan, organised by Association of Food Scientists & Technologists (India), CSIR-CFTRI campus, Mysore 570020, India.
4. **Gowthami G. A**., and Gunashree B. S., (2022). A **Poster** was presented on the topic entitled "**Optimization of chemical parameters for the production of carotenoids from *Rhodotorula minuta*"** at 2 days National Conference on "Advances in Microbial Biotechnology; Current Trends and Future Prospects". Organized by the Department of Microbiology, Biotechnology & Food Technology in Association with Microbiologist Society, India (MSI) held on the 28th and 29th April.
5. **Gowthami G. A**., and Gunashree B. S., (2022). **Oral Presentation** was made on **"Optimization of carbon and nitrogen sources for lipids and exopolysaccharide production from *Rhodotorula minuta* ON307085"** in a 2 days National Conference on "Integrated Microbial Technology for Sustainable Future" – (IMTSF-2022). Organized by the Department of Studies and Research in Microbiology, Mangalore University, PG Centre, Chikka Aluvara, Kodagu 571232 held on the 7th and 8th of July.
6. **Gowthami G. A**., and Gunashree B. S., (2022). **Oral Presentation** was made on **"Optimization of physical parameters for Lipids and Exopolysaccharide production from *Rhodotorula minuta*"** at one day National Conference on "Recent Advancements in Chemistry" organized by the Department of Chemistry, Field Marshal K.M. Cariappa College, Madikeri held on the 23rd of November.

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